

METHODS OF CLONING GOATS USING METAPHASE II OOCYTES

RELATED APPLICATION(S)

This application claims the benefit of U.S. Provisional Application No. 60/131,057, filed on April 26, 1999, entitled, "Methods of Cloning Goats Using Metaphase II Oocytes," by Baguisi, *et al.*; U.S. Provisional Application No. 60/131,328, filed April 26, 1999, entitled, "Transgenic and Cloned Mammals," by Baguisi, *et al.*, and U.S. Provisional Application No. (not known, Attorney Docket No., 10275-101-001), filed November 2, 1998, entitled, "Transgenic and Cloned Mammals," by Echelard, *et al.*, the entire teachings of which are incorporated herein by reference.

GOVERNMENT SUPPORT

The invention was supported, in part, by a grant 1R43HD/GM35395-01 from National Institutes of Health, Institute of Child Health and Development. The Government has certain rights in the invention.

15 BACKGROUND OF THE INVENTION

Recent developments in biotechnology have allowed for the modification of an animal genome through the use of transgenic technology. This advancement in technology allows for the expression of biomedical proteins in domesticated animals facilitating low cost production of high quantities of valuable proteins, including those proteins important in the pharmaceutical area. Houdebine, *Reprod. Nutr. Dev.*, 35:609-617 (1995); Maga *et al.*, *Bio/Technology*, 13: 1452-1457 (1995); Echelard, *Curr Op Biotechnol*, 7:536-540 (1996); Young *et al.*, *Biopharm*, 10:34-38 (1997). Transgenic technology is applicable and attractive for proteins that traditionally have been very difficult to isolate and purify from bioreactors and cell/tissue culture systems. The ability to introduce a transgenic nucleotide sequence into a host animal thereby allowing for the production and expression of this transgenic

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nucleotide sequence obviates difficulties observed using traditional methods for obtaining desirable proteins. For example, if the transgenic nucleotide sequence is introduced into a procaryotic bioreactor, such as *E. coli*, is a eucaryotic nucleotide sequence, the introns (or intervening sequences) within the transgenic nucleotide
5 sequence would not be spliced-out using the *E. coli* system. By cloning eucaryotic transgenic nucleotide sequences into animal hosts these nucleotide sequences can undergo both transcriptional and postranslational modifications which are often necessary for the full and viable function of a mature protein.

Previous studies have indicated that dairy goats are ideal for the transgenic
10 production of therapeutic recombinant proteins. The average milk output is 600 to 800 liters per lactation. With herds of a manageable size and at concentrations of approximately 1 to 5 grams per liter reproducibly achieved with various animal models, yields of transgenic protein to obtain 100 to 300 kg of purified product per year are achievable. Gordon *et al.*, *Bio/Technology*, 5:1183-1187 (1987); Meade *et al.*, *Bio/Technology*, 8:443-446 (1990); Ebert *et al.*, *Bio/Technology*, 9:A35-A38
15 (1991); Simons *et al.*, *Nature*, 328:530-532 (1987); Wright *et al.*, *Bio/Technology*, 9:801-834 (1991); Velander *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:12003-12007 (1992); Hansson, *et al.*, *J. Biol. Chem.*, 269:5358-5363 (1994); Hurwitz, *et al.*, *Transgenic Res.*, 3:365-375 (1994).

20 Currently, there are very few reliable methods of producing transgenic goats. One such method is pronuclear microinjection. Using pronuclear microinjection methods, transgene integration into the genetic make-up of, for example in goats, occurs in 1 to 3 percent of all microinjected embryos. Ebert *et al.*, *Theriogenology*, 39:121-135 (1993). Recently it was reported that most embryonic stem cells could
25 be isolated and propagated *in vitro* and genetically modified which would contribute to the germ line of a host embryo. Evans *et al.*, *Nature*, 292:154-156 (1981); Martin, *Proc. Natl. Acad. Sci. USA*, 78:7634-7638 (1981); Bradley *et al.*, *Nature*, 309:255-256 (1984). Others have used murine embryonic stem cells to modify single targeted genes. Mansour *et al.*, *Nature*, 336:348-352 (1988); McMahon *et al.*,
30 *Cell*, 62: 1073-1085 (1990); recently reviewed in Bronson *et al.*, *J. Biol. Chem.*, 269: 27155-27158 (1994); Rossant, *et al.*, *Nat. Med.*, 6:592-594 (1995).

Studies conducted using the mouse have indicated the utility of this powerful technology, yet a need still exists for methods of obtaining cloned and transgenic goats.

SUMMARY OF THE INVENTION

5 The present invention is based in part on the discovery that cloned and transgenic goats can be produced by the introduction of a caprine donor cell nuclear genome into an enucleated caprine metaphase oocyte.

 In one embodiment of the invention, the method of cloning a goat by combining a caprine donor cell genome with an enucleated, metaphase caprine
10 oocyte is described. In this embodiment the caprine donor cell and enucleated metaphase caprine oocyte are obtained from caprine sources. Preferably, the caprine donor cells that are used in this embodiment are harvested from a synchronous culture system. This synchronous culture system preferably contains a majority of donor cells in G₁ phase of the cell cycle. However, in this embodiment the donor
15 cells can be in G₀, S, G₂/M phase of the cell cycle. The G, S and M donor cells can either be adult or embryonic donor cells. Both fibroblast and epithelial donor cells are encompassed within this embodiment. The caprine donor cells can be somatic cells, germ cells or stem cells. The caprine oocyte by being in a metaphase stage of the meiotic cell cycle is in an arrested state. The oocyte is preferably in metaphase
20 II of meiotic cell division. Preferably, the caprine oocyte is enucleated by virtue of not having a nucleus present. Alternatively, the caprine oocyte can be rendered functionally enucleated by virtue of having its nuclear genome rendered incapable of functioning. The genome of the caprine donor cell can be introduced into the enucleated caprine oocyte by a variety of means. For example, the caprine donor
25 cell's genome can be introduced into the caprine enucleated oocyte by a microinjection, or by fusing the two cells together. The introduction of the donor cell's genome into the enucleated oocyte produces a nuclear transfer embryo. This caprine nuclear transfer embryo proceeds through gestation thereby forming a cloned goat. The cloned goat following parturition can produce one, or more,
30 desired proteins which can then be recovered and used, for example, in therapeutic regimes. For example, if the cloned goat is a female goat, then that cloned female

goat can be induced to lactate and thereby, produce milk. Assuming that the transgenic nucleotide sequence is specific for the mammary gland, then upon lactation the cloned female will produce the expressed transgenic product in the milk. The cloned goat can be a male goat.

- 5 In another embodiment of the present invention, a method of producing a transgenic goat by combining a genetically engineered genome from a caprine donor cell with an enucleated metaphase caprine oocyte is described. The caprine donor cell's genome can contain a transgenic nucleotide sequence. This transgenic nucleotide sequence can involve homologous recombination which knocks out,
- 10 knocks in, or otherwise disrupts the expression of the intrinsic donor cell's gene. This transgenic nucleotide sequence can be a sequence encoding a hormone, an immunoglobulin, a plasma protein, an enzyme, a secreted protein, a neuropeptide, neurotransmitter, a non-secreted protein, or a structural protein. This transgenic nucleotide sequence can either be a human or non-human nucleotide sequence.
- 15 Preferably the transgenic nucleotide sequence contains an operatively linked promoter nucleotide sequence.

- In still another embodiment of the present invention, a method of producing a heterologous protein in a goat is described. A nuclear transfer embryo is formed by introducing a genetically engineered caprine genome containing a transgene
- 20 encoding a heterologous protein into a enucleated caprine oocyte that is in a metaphase stage of meiotic cell division. The nuclear transfer embryo can be implanted into a suitable goat female where it can undergo gestation. Following parturition the goat can produce the heterologous protein. The heterologous protein can then be recovered from the developed goat.

- 25 The present invention encompasses methods for cloning goats. The methods described herein involve the introduction of a genome from a caprine donor cell into an enucleated caprine oocyte that is in a metaphase stage of meiotic cell division. Nuclear transfer of a donor cell allows for the selection of the appropriate transgenic cell line for the generation of cloned transgenic embryos. This is particularly
- 30 important in the case where several proteins are to be co-expressed by the transgenic animal, for example, using the mammary gland, for example, in the transgenic production of a recombinant monoclonal antibody in the milk. The heavy chain and

light chain transgenes ideally should be expressed in the same secretory cells of the mammary epithelium at equivalent levels for the efficient production of intact antibodies. In addition, transgenes expressing each protein should be co-integrated in the same locus to favor equivalent expression and avoid segregation of heavy
5 chain and light chain transgenes during propagation.

In one embodiment, the caprine oocyte is combined with a caprine donor cell by fusion and is activated. In a preferred embodiment, the caprine oocyte is combined with a caprine donor cell by simultaneous fusion and activation. In another preferred embodiment, the caprine oocyte is combined with a caprine donor
10 cell by fusion and is then activated. In still another preferred embodiment, the caprine oocyte is first activated and is then combined with the caprine donor cell by fusion.

The generation of transgenic animals that have completely identical genetic backgrounds also enhances the possibility of studying the expression in secretion
15 characteristics of recombinant proteins by, for example, the mammary gland. For example, the availability of several completely identical transgenic goat females producing recombinant human proteins will help to determine the extent of variation in, for example, the carbohydrate structure of a particular protein. Thus, it may be feasible to improve the characteristics of the recombinant proteins produced in
20 transgenic animal systems by varying environmental factors, for example, nutrition or to decrease the milk volume yield of lactation-induction protocols to diminish further the time necessary to obtain adequate amounts of recombinant proteins for preclinical or clinical programs. Thus, the development of methods for cloning goats can lead to the advancement in the technology of producing recombinant
25 proteins.

DETAILED DESCRIPTION OF THE INVENTION

The features and other details of the invention will now be more particularly described and pointed out below and in the claims. It will be understood that the particular embodiments of the invention are shown by way of illustration and not as
30 limitations of the invention. The principal feature of this invention can be employed in various embodiments without departing from the scope of the invention.

The present invention is directed to methods for cloning goats. The instant invention is based in part on the discovery that cloned and transgenic goats can be produced by the introduction of a caprine donor cell's nuclear genome into an enucleated caprine oocyte that is in a metaphase stage of meiotic cell division. In one embodiment of the invention, a method of cloning a goat by combining a genome from a caprine donor cell with a enucleated caprine oocyte that is in a metaphase stage of meiotic cell cycling in the production of a nuclear transfer embryo which is suitable for gestation is described. The caprine donor cell can be a somatic cell, germ cell or a stem cell.

10 The term "somatic cell" as used herein refers to a differentiated cell. The cell can be a somatic cell or a cell that is committed to a somatic cell lineage. Alternatively, any of the methods described herein can utilize a diploid stem cell that gives rise to a germ cell in order to supply the genome for producing a nuclear transfer embryo. The somatic cell can originate from a goat or from a cell and/or tissue culture system. If taken from a goat, the goat can be at any stage of development, for example, an embryo, a fetus or an adult. In one embodiment, caprine embryonic somatic cells are used. Embryonic cells can include embryonic stem cells as well as embryonic cells committed to a somatic cell lineage. Such cells can be obtained from the endoderm, mesoderm or ectoderm of the embryo.

15 Preferably, the embryonic cells are committed to a somatic cell lineage. Embryonic cells committed to a somatic cell lineage refer to cells isolated on or after approximately day ten of embryogenesis. However, cells can be obtained prior to day ten of embryogenesis. If a cell line is used as a source for a nuclear genome, then primary cells are preferred. The term "primary cell line" as used herein includes

20 primary cells as well as primary derived cell lines.

Suitable caprine somatic cells include fibroblasts (for example, primary fibroblasts), muscle cells, cumulous cells, neural cells, and mammary cells. Other suitable cells include hepatocytes and pancreatic islets. The genome of the caprine somatic cell can be the naturally occurring genome, for example, for the production of cloned goats, or the genome can be genetically altered to comprise a transgenic sequence, for example, for the production of transgenic cloned goats.

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Caprine somatic cells can be obtained by, for example, disassociation of tissue by mechanical (e.g., chopping, mincing) or enzymatic means (e.g., trypsinization) to obtain a cell suspension followed by culturing the cells until a confluent monolayer is obtained. The somatic cells can then be harvested and
5 prepared for cryopreservation, or maintained as a stalk culture. The isolation of somatic cells, for example, fibroblasts, is described herein.

The caprine donor cell can be a quiescent or non-quiescent donor cell. The term "non-quiescent", as used herein, refers to a cell in the mitotic cell cycle. The mitotic cell cycle has four distinct phases, G_1 , S, G_2 and M. The commitment event
10 in the cell cycle, called START (or restriction point), takes place during the G_1 phase. "START" as used herein refers to late G_1 stage of the cell cycle prior to the commitment of a cell proceeding through the cell cycle. The decision as to whether the cell will undergo another cell cycle is made at START. Once the cell has passed through START, it passes through the remainder of the G_1 phase (i.e., the pre-DNA
15 synthesis stage). The S phase is the DNA synthesis stage, which is followed by the G_2 phase, the stage between synthesis and mitosis. Mitosis takes place during the M phase. If prior to START, the cell does not undergo another cell cycle, the cell becomes arrested. In addition, a cell can be induced to exit the cell cycle and become quiescent. A "quiescent" cell, also referred to as a cell in G_0 phase, refers to
20 a cell which is not in any of the four phases of the cell cycle. Preferably, the donor cell is a cell in the G_0 phase or the G_1 phase of the mitotic cell cycle.

Using caprine donor cells at certain phases of the cell cycle, for example, G_0 or G_1 phase, can allow for synchronization between the caprine oocyte and the genome of a donor cell. In one embodiment, an oocyte in metaphase II fused, for
25 example, by simultaneous activation and fusion, with the genome of a caprine donor cell in G_0 or G_1 provides a synchronization of the cell cycles between the oocyte and donor nuclei. In another embodiment, the donor cell is fused with the activated enucleated oocyte and then activated a second time. In still another embodiment, the activated oocyte is fused with a donor cell without further activation.

30 Methods of determining which phase of the cell cycle a cell is in are known to those skilled in the art, for example, U.S. Patent No. 5,843,705 to DiTullio *et al.*, Campbell, K. H. S., *et al.*, *Embryo Transfer Newsletter*, vol. 14(1):12-16 (1996),

Campbell, K. H. S., *et al.*, *Nature*, 380:64-66 (1996), Cibelli, J. B., *et al.*, *Science*, 280:1256-1258 (1998), Yong, Z. and L. Yuqiang, *Biol. of Reprod.*, 58:266-269 (1998) and Wilmut, I., *et al.*, *Nature*, 385:810-813 (1997), the teachings of which are herein incorporated by reference in their entirety. For example, as described
5 below in the Examples, various markers are present at different stages of the cell cycle. Such markers can include cyclins D 1, 2, 3 and proliferating cell nuclear antigen (PCNA) for G₁, and BrDu to detect DNA synthetic activity. In addition, cells can be induced to enter the G₀ stage by culturing the cells on a serum-deprived medium. Alternatively, cells in G₀ stage can be induced to enter into the cell cycle,
10 that is, at G₁ stage by serum activation.

Methods for generating transgenic goats which can be used as a source of caprine donor cells in the instant invention are known to those of skill in the art. Such methods can involve introducing DNA constructs into the germ line of a goat to make a transgenic goat. For example, one or several copies of the construct may
15 be incorporated into the genome of a goat embryo by standard transgenic techniques.

Although goats are a preferred source of genetically engineered donor cells, other goats can be used. Preferred non-human mammals are ruminants, for example, cows, sheep, camels, pigs, oxen, horses, llamas and mice. Goats of Swiss origin, for example, the Alpine, Saanen and Toggenburg bread goats, are useful in the methods
20 described herein. The animal used as a source of genetically engineered cells will depend on the transgenic animal to be obtained by the methods of the invention as, by way of example, a goat genome should be introduced into a goat enucleated oocyte in a metaphase stage of meiotic cell division.

The somatic cells for use in the current invention are obtained from a
25 transgenic goat. Methods of producing transgenic goats are known to those of skill in the art. For example, a transgene can be introduced into the germ line of a goat by microinjection as described, for example, in Ebert *et al.*, 12:699 (1994), the teachings of which are herein incorporated by reference in its entirety.

Other transgenic goats to be used as a source of genetically engineered
30 somatic cells can be produced by introducing a transgene into the germ line of the goat. Embryonal target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending upon the stage of

development of the embryonal target cell. The specific line(s) of any animal used to practice this invention are selected for general good health, good embryo yields, good pronuclear visibility in the embryo, and good reproductive fitness. In addition, the haplotype is a significant factor.

5 Genetically engineered caprine donor cells for use in the instant invention can be obtained from a cell line into which a nucleic acid of interest, for example, a nucleic acid which encodes a protein, has been introduced.

 A construct can be introduced into a cell via conventional transformation or transfection techniques. As used herein, the terms "transfection" and
10 "transformation" include a variety of techniques for introducing a transgenic sequence into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE dextrane-mediated transfection, lipofection, or electroporation. In addition, biological vectors, for example, viral vectors can be used as described below. Suitable methods for transforming or transfecting host
15 cells can be found in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual In Second Edition*, Cold Spring Harbor Laboratory, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989). Two useful and practical approaches for introducing genetic material into a cell are electroporation and lipofection.

 The DNA construct can be stably introduced into a donor cell line by
20 electroporation using the following protocol: donor cells, for example, embryonic fibroblasts, are resuspended in phosphate buffer saline (PBS) at about 4×10^6 cells per mL. Fifty micrograms of linearized DNA is added to the 0.5 mL cell suspension, and the suspension is placed in a 0.4 cm electrode gap cuvette. Electroporation is performed using a BioRad Gene Pulser (Bio Rad) electroporator
25 with a 330 volt pulse at 25 mA, 1000 microFarad and infinite resistance. If the DNA construct contains a neomycin resistance gene for selection, neomycin resistant clones are selected following incubation where 350 mg/mL of G418 (GIBCO BRL) for fifteen days.

 The DNA construct can be stably introduced into a donor somatic cell line
30 by lipofection using a protocol such as the following: about 2×10^5 cells are plated into a 3.5 cm well and transfected with 2 mg of linearized DNA using LipfectAMINE® (GIBCO BRL). Forty-eight hours after transfection, the cells are

split 1: 1000 and 1: 5000 and if the DNA construct contains a neomycin resistance gene for selection, G418 is added to a final concentration of 0.35 mg/mL.

Neomycin resistant clones are isolated and expanded for cryopreservation as well as nuclear transfer.

5 It is often desirable to express a protein, for example, a heterologous protein, in a specific tissue or fluid, for example, the milk of a transgenic goat. The heterologous protein can be recovered from the tissue or fluid in which it is expressed. For example, it is often desirable to express the heterologous protein in milk. Methods for producing a heterologous protein under the control of a milk-specific promoter is described below. In addition, other tissue-specific promoters, as well as, other regulatory elements, for example, signal sequences and sequences which enhance secretion of non-secreted proteins, are described below. The transgenic product (e.g., a heterologous protein) can be expressed, and therefore, recovered in the goat's blood, urine, hair, skin, muscle, or viscera (or a tissue component thereof) including, but not limited to, brain, heart lung, kidney, pancrease, gall bladder, liver, stomach, eye, colon, small intestine, bladder, uterus and testes. Recovery of a transgenic product from these tissues are well known to those skilled in the art, see, for example, Ausubel, F. M., *et al.*, (eds), *Current Protocols in Molecular Biology*, vol. 2, ch. 10 (1991), the entire teachings of which are herein incorporated by reference.

Useful transcriptional promoters are those promoters that are preferentially activated in mammary epithelial cells, including promoters that control the genes encoding protein such as caseins, β -lactoglobulin (Clark *et al.*, *Bio/Technology*, 7:487-492 (1989)), whey acid protein (Gordon *et al.*, *Bio/Technology*, 5:1183-1187 (1987)), and lactalbumin (Soulier *et al.*, *Febs Letts.*, 297:13 (1992)). Casein promoters may be derived from the alpha, beta, gamma, or kappa casein genes of any animal species; a preferred promoter is derived from the goat β -casein gene (Ditullio, *Bio/Technology*, 10:74-77 (1992)). Milk specific protein promoter or the promoters that are specifically activated in mammary tissue can be derived from cDNA or genomic sequences.

DNA sequence information is available for the mammary gland's specific genes listed above, in at least one, and often in several organisms. See, for example,

Richards *et al.*, *J. Biol. Chem.*, 256:526-532 (1981) (α -Lactalbumin rat); Campbel *et al.*, *Nucleic Acids Res.*, 12:8685-8697 (1984) (rat WAP); Jones *et al.*, *J. Biol. Chem.*, 260:7042-7050 (1985) (rat β -Casein); Yu-Lee and Rosen, *J. Biol. Chem.*, 258:10794-10804 (1983) (rat α -Casein); Hall, *Bio. Chem. J.*, 242:735-742 (1987);

5 (α -Lactalbumin human); Stewart, *Nucleic Acids Res.*, 12:389 (1984) (Bovine α S1 and κ 1 Casein, cDNAs); Gorodetsky *et al.*, *Gene*, 66:87-96 (1988) (Bovine β -Casein); Alexander *et al.*, *Eur. J. Biochem.*, 178:395-401 (1988) (Bovine and κ -Casein); Brignon *et al.*, *Febs Let.*, 188:48-55 (1977) (Bovine α S2 Casein); Gamieson *et al.*, *Gene*, 61:85-90 (1987); Ivanov *et al.*, *Biol. Chem. Hopp-Seylar*,

10 369:425-429 (1988); Alexander *et al.*, *Nucleic Acid Res.*, 17:6739 (1989) (Bovine β -Lactoglobulin); Vilotte *et al.*, *Biochimie*, 69:609-620 (1987) (Bovine α -Lactalbumin), the teachings of which are here incorporated in their entirety by reference.

The structure and function of the various milk protein genes are reviewed by

15 Mercier & Vilotte, *J. Dairy Sci.*, 76:3079-3098 (1993), the teachings of which are incorporated herein by reference and its entirety. If additional flanking sequences are useful in optimizing expression of the heterologous protein, such sequences can be cloned using the existing sequences as probes. Mammary gland specific regulatory sequences from different organisms can be obtained by screening libraries

20 from such organisms using known cognate nucleotide sequences, or antibodies to cognate proteins as probes.

Useful signal sequences such as milk specific signal sequences or other signal sequences which result in the secretion of eukaryotic or prokaryotic proteins can be used. Preferably, the signal sequence is selected from milk specific signal

25 sequences, that is, it is from a gene which encodes a product secreted into milk. Most preferably, the milk specific signal sequence is related to the milk specific promoter used in the construct, which is described below. The size of the signal sequence is not critical. All that is required is that the sequence be of a sufficient size to effect secretion of the desired recombinant protein, for example, in the

30 mammary tissue. For example, signal sequences from genes coding for caseins, for example, α , β , γ or κ caseins and the like can be used. A preferred signal sequence is the goat β -casein signal sequence. Signal sequences from other secreted proteins,

for example, proteins secreted by kidney cells, pancreatic cells, or liver cells, can also be used. Preferably, the signal sequence results in the secretion of proteins into, for example, urine or blood.

A non-secreted protein can also be modified in such a manner that it is
5 secreted such as by inclusion in the protein to be secreted all or part of the coding sequence of a protein which is normally secreted. Preferably, the entire sequence of the protein which is normally secreted is not included in the sequence of the protein but rather only a sufficient portion of the amino terminal end of the protein which is normally secreted to result in secretion of the protein. For example, a portion which
10 is not normally secreted is fused (usually at its amino terminal end) to an amino terminal portion of the protein which is normally secreted.

In one aspect, the protein which is normally secreted is a protein which is normally secreted in milk. Such proteins include proteins secreted by mammary epithelial cells, milk proteins such as caseins, β -lactoglobulin, whey acid protein,
15 and lactalbumin. Casein proteins including, alpha, beta, gamma or kappa casein genes of any mammalian species. The preferred protein is β -casein, for example, goat β -casein. Sequences which encode the secreted protein can be derived from either cDNA or genomic sequences. Preferably, they are of genomic origin, and include one or more introns.

20 Other tissue specific promoters which provide expression in a particular tissue can be used. Tissue specific promoters are promoters which are expressed more strongly in a particular tissue than in others. Tissue specific promoters are often expressed exclusively in the specific tissue.

Tissue specific promoters which can be used include: a neural-specific
25 promoter, for example, nestin, Wnt-1, Pax-1, Engrailed-1, Engrailed-2, Sonic-hedgehog; a liver specific promoter, for example, albumin, alpha-1, antitrypsin; a muscle-specific promoter, for example, myogenin, actin, MyoD, myosin; an oocyte specific promoter, for example, ZP1, ZP2, ZP3; a testis specific promoter, for example, protamine, fertilin, synaptonemal complex protein-1; a blood specific
30 promoter, for example, globulin, GATA-1, porphobilinogen deaminase; a lung specific promoter, for example, surfactin protein C; a skin or wool specific promoter, for example, keratin, elastin; endothelium-specific promoter, for example,

TIE-1, TIE-2; and a bone specific promoter, for example, BMP. In addition, general promoters can be used for expression in several tissues. Examples of general promoters, include β -actin, ROSA-21, PGK, FOS, c-myc, Jun-A, and Jun-B.

A cassette which encodes a heterologous protein can be assembled as a
5 construct which includes a promoter for a specific tissue, for example, for mammary epithelial cells, a casein promoter. The construct can also include a 3' untranslated region downstream of the DNA sequence coding for the non-secreted proteins. Such regions can stabilize the RNA transcript of the expression system and thus increase the yield of desired protein from the expression system. Among the 3' untranslated
10 regions useful in the constructs for use in the invention are sequences that provide a polyA signal. Such sequences may be derived, for example, from the SV40 small t antigen, the casein 3' untranslated region or other 3' untranslated sequences well known in the art. In one aspect, the 3' untranslated region is derived from a milk specific protein. The length of the 3' untranslated region is not critical but the
15 stabilizing effect of its polyA transcript appears imported in stabilizing the RNA of the expression sequence.

Optionally, the construct can include a 5' untranslated region between the promoter and the DNA sequence encoding the signal sequence. Such untranslated regions can be from the same control region as that from which the promoter is
20 taken or can be from a different gene, for example, they may be derived from other synthetic, semisynthetic or natural sources. Again, there specific length is not critical, however, they appear to be useful in improving the level of expression.

The construct can also include about 10%, 20%, 30% or more of the N-terminal coding region of a gene preferentially expressed in mammary epithelial
25 cells. For example, the N-terminal coding region can correspond to the promoter used, for example, a goat β -casein N-terminal coding region.

The construct can be prepared using methods known to those skilled in the art. The construct can be prepared as part of a larger plasmid. Such preparation allows the cloning and selection of the correct constructions in an efficient manner.
30 The construct can be located between convenient restrictions sites on the plasmid so that they can be easily isolated from the remaining plasmid sequences for incorporation into the desired animal.

Transgenic sequences encoding heterologous proteins can be introduced into the germ line of a goat or can be transfected into a cell line to provide a source of genetically engineered caprine donor cells as described above. The protein can be a complex or multimeric protein, for example, a homo- or heteromultimeric proteins.

5 The protein can be a protein which is processed by removing the N-terminus, C-terminus or internal fragments. Even complex proteins can be expressed in active form. Protein encoding sequences which can be introduced into the genome of a goat include glycoproteins, neuropeptides, immunoglobulins, enzymes, peptides and hormones. The protein may be a naturally occurring protein or a recombinant

10 protein for example, a fragment or fusion protein, (e.g., an immunoglobulin fusion protein or a mutant). The protein encoding nucleotide sequence can be human or non-human in origin. The heterologous protein may be a potential therapeutic or pharmaceutical agent such as, but not limited to, alpha-1 proteinase inhibitor, alpha-1 antitrypsin, alkaline phosphatase, angiogenin, antithrombin III, any of the blood

15 clotting factors including Factor VIII, Factor IX, and Factor X chitinase, erythropoietin, extracellular superoxide dismutase, fibrinogen, glucocerebrosidase, glutamate decarboxylase, human growth factor, human serum albumin, immunoglobulin, insulin, myelin basic protein, proinsulin, prolactin, soluble CD 4 or a component or complex thereof, lactoferrin, lactoglobulin, lysozyme,

20 lactalbumin, tissue plasminogen activator or a variant thereof. Immunoglobulin particularly preferred protein. Examples of immunoglobulins include IgA, IgG, IgE, IgM, chimeric antibodies, humanized antibodies, recombinant antibodies, single chain antibodies and anti-body protein fusions.

Nucleotide sequence information is available for several of the genes

25 encoding the heterologous proteins listed above, in at least one, and often in several organisms. See, for example, Long *et al.*, *Biochem.*, 23(21):4828-4837 (1984) (Alpha-1 antitrypsin); Mitchell *et al.*, *Proc. Natl. Acad. Sci. USA*, 83:7182-7186 (1986) (Alkaline phosphatase); Schneider *et al.*, *Embo J.*, 7(13): 4151-4156 (1988) (Angiogenin); Bock *et al.*, *Biochem.*, 27 (16):6171-6178 (1988) (Antithrombin);

30 Olds *et al.*, *Br. J. Haematol.*, 78(3): 408-413 (1991) (Antithrombin III); Lyn *et al.*, *Proc. Natl. Acad. Sci. USA*, 82(22):7580-7584 (1985) (erythropoietin); U.S. Pat No. 5,614,184 (erythropoietin) Horowitz, *et al.*, *Genomics*, 4(1):87-96 (1989)

(Glucocerebrosidase); Kelly *et al.*, *Ann. Hum. Genet.*, 56(3):255-265 (1992)
 (Glutamate decarboxylase); U.S. Pat No. 5,707,828 (human serum albumin); U.S.
 Pat. No. 5,652,352 (human serum albumin); Lawn *et al.*, *Nucleic Acid Res.*,
 9(22):6103-6114 (1981) (human serum albumin); Kamholz *et al.*, *Prot. Natl.*
 5 *Acad. Sci. USA*, 83(13):4962-4966 (1986) (myelin basic protein); Hiraoka *et al.*,
Mol. Cell Endocrinol., 75(1):71-80 (1991) (prolactin); U.S. Pat. No. 5,571,896
 (lactoferrin); Pennica *et al.*, *Nature*, 301(5897):214-221 (1983) (tissue plasminogen
 activator); Sarafanov *et al.*, *Mol. Biol.*, 29: 161-165 (1995), the teachings of which
 are herein incorporated by reference in their entirety.

10 Oocytes used in the current invention are caprine oocytes that are in a
 metaphase stage of meiotic cell division. Preferably, the oocytes are in metaphase II
 stage of meiotic cell division. Oocytes that are in metaphase can be differentiated
 from telophase oocytes based on the presence of having only one polar body.
 Additionally, metaphase II oocytes can be differentiated from telophase oocytes
 15 based on biochemical and/or developmental differences. For example, oocytes in
 metaphase II can be in an arrested state, whereas telophase oocytes are in an active
 state. Oocytes can be obtained from a caprine donor during the goat's reproductive
 cycle. For example, at given times following ovulation, a significant percentage of
 the oocytes. Additionally, oocytes can be obtained and then induced to mature *in*
 20 *vitro* to the arrested metaphase II stage.

Oocytes can be collected from a female goat following super ovulations.
 Briefly, oocytes can be recovered surgically by flushing the caprine oocytes from the
 oviduct of a female goat. Methods of inducing super ovulations in goats and the
 collection of the oocytes are described herein. Preferably the cell stage of the
 25 oocytes correlates to the stage of the cell cycle of the caprine donor cell. This
 correlation between the meiotic stage of the caprine oocyte and the mitotic stage of
 the caprine donor cell is referred to herein as "synchronization." For example, a
 caprine oocyte in metaphase fused with the genome of a caprine donor cell in G₀ or
 G₁ by, for example, simultaneous fusion and activation, can mimic the events
 30 occurring during fertilization.

The caprine oocyte should be enucleated such that the endogenous genome
 of the oocyte is incapable of functioning, for example, replicating or synthesizing

DNA. Preferably the genome of the caprine oocyte is removed from the oocyte. Alternatively, the caprine oocyte can be rendered functionally incapable of performing the functions of an intact genome. This can be accomplished by inactivating the DNA within the caprine oocyte by, for example, irradiation, for example by x-ray irradiation, or laser irradiation; chemical inactivation, or the like.

One method of enucleating the genome of a caprine oocyte is to insert a micropipette or needle into the zona pellicuda in order to remove nuclear material from the oocyte. Methods of enucleating a caprine oocyte are described in further detail in the Examples. The caprine oocyte can be rendered functionally inactive by irradiating the endogenous nuclear material in the oocyte. Methods of using irradiation are known to those in the art and are described, for example, in Bradshaw *et al.*, *Molecul. Reprod. Dev.*, 41:503-512 (1995), the teachings of which are incorporated herein by reference in its entirety. The caprine oocyte can be rendered functionally inactive also by chemical methods. Methods of chemically inactivating the DNA are known to those of skill in the art. For example, chemical inactivation can be preformed using the etoposide cycloheximide method as described in Fulkaj and Moore, *Molecul. Reprod. Dev.*, 34:427-430 (1993), the teachings of which are incorporated herein by reference in its entirety.

Methods described herein include the introduction of a functional nuclear genome from a caprine donor cells into an enucleated caprine oocyte that is in a metaphase stage of the meiotic cell cycle to form a nuclear transfer embryo. The functional nuclear genome donated from a caprine donor cell directs the development of a cloned or transgenic goat which arises from the nuclear transfer embryo. Methods which result in the transfer of an essentially intact nuclear genome to the caprine oocyte can be used.

In one embodiment of the invention, the nuclear genome of the caprine donor cell is injected into the caprine oocyte by employing a microinjector (i.e., micropipette or needle). The nuclear genome of the caprine donor cell, for example, a somatic cell, is extracted using a micropipette or needle. Once extracted, the donor's nuclear genome can then be placed into the caprine oocyte by inserting the micropipette, or needle, into the oocyte and releasing the nuclear genome of the donor's cell. McGrath, J. and D. Solter, *Science*, 226:1317-1319 (1984).

In one embodiment of the present invention, fusion of the caprine donor cell with a caprine oocyte can be preformed by, for example, electrofusion, viral fusion, liposomal fusion, biochemical reagent fusion (for example phytohemagglutinin (PHA) protein), or chemical fusion (for example polyethylene glycol (PEG) or ethanol). The nucleus of the caprine donor cell can be deposited within the zona pelliduca which contains the caprine oocyte. The steps of fusing the nucleus with the caprine oocyte can then be performed by applying an electric field which will also result in a second activation of the oocyte. Activation refers to the beginning of embryonic development, for example, replication and DNA synthesis. Activation can be induced by for example electric shock (e.g., in electrofusion), the use of ionophores or ethanol activation. The caprine oocytes used in the current invention are oocytes that are in a metaphase stage of cell meiotic cycle.

A nuclear transfer embryo can result from electrofusion which allows for the fusion of the caprine donor cell with an enucleated caprine oocyte in metaphase II stage of meiotic cell division. Chambers, such as the BTX 200 Embryomanipulation System for carrying out electrofusion are commercially available from for example BTX, San Diego.

The nuclear transfer embryo can be activated by ionophore activation. Using a ionophore, for example, a calcium ionophore. As the free calcium concentration in the cell increases during exposure to the ionophore, the caprine oocyte is activated following reduction in MPF (maturation promoting factor) activity. Such methods of activation are described in U.S. Patent No. 5,496,720, the teachings of which are herein incorporated by reference in its entirety.

Prior to, or following, enucleation, an oocyte in metaphase II can be activated with ethanol according to the ethanol activation treatment as described in Presicce and Yang, *Mol. Reprod. Dev.*, 37:61-68 (1994); and Bordignon & Smith, *Mol. Reprod. Dev.*, 49:29-36 (1998), the teachings of which are herein incorporated by referenced in their entirety.

A nuclear transfer embryo of the current invention can be transferred into a recipient female goat and allowed to develop into a cloned or transgenic goat. For example, the nuclear transfer embryo can be transferred via the fimbria into the oviductal lumen of each recipient goat female as described in the Examples. In

addition, methods of transferring an embryo to a recipient are well known to those skilled in the art and are described in Ebert *et al*, *Bio/Technology*, 12:699 (1994), the teachings of which are incorporated herein by reference in its entirety. The nuclear transfer embryo can be maintained in a culture system until at least first cleavage (2-cell stage) up to the blastocyst stage, preferably the embryos are transferred at the 2-cell or 4-cell stage. Various culture media for embryo development are known to those skilled in the art. For example, the nuclear transfer embryo can be co-cultured with oviductal epithelial cell monolayer derived from a goat. For example methods of obtaining goat oviductal epithelial cells (GOEC) maintaining the cells in a co-culture are described in the Examples below.

A transgenic protein can be produced in the transgenic cloned goat at relatively high concentrations and in large volumes, for example in milk, providing continuous high level output of normally processed protein that is easily harvested from a renewable resource. There are several different methods known in the art for isolation of proteins for milk.

Milk proteins usually are isolated by a combination of processes. Raw milk first is fractionated to remove fats, for example by skimming, centrifugation, sedimentation, (H.E. Swaisgood, Development in Dairy Chemistry, I: Chemistry of Milk Protein, Applied Science Publishers, NY 1982, the teachings of which are incorporated herein by reference in its entirety), acid precipitation (U.S. Patent No. 4,644,056, the teachings of which are incorporated herein by reference in its entirety) or enzymatic coagulation with rennin or chymotrypsin (Swaisgood, *ibid.*). Next the major milk proteins may be fractionated into either a clear solution or a bulk precipitate from which this specific protein of interest may be readily purified.

French Patent No. 2487642 describes the isolation of milk proteins from skim milk or whey by performing ultra filtration in combination with exclusion chromatography or ion exchange chromatography, the teachings of which are herein incorporated by referenced in its entirety. Whey is first produced by removing the casein by coagulation with rennet or lactic acid. U.S. Patent No. 4,485,040 describes the isolation of an α -lactoglobulin-enriched product in the retentate from whey by two sequential ultra filtration steps, the teachings of which are incorporated herein by reference in its entirety. U.S. Patent No. 4,644,056 provides a method for

purifying immunoglobulin from milk or colostrum by acid precipitation at pH 4.0-5.5, is sequential cross-flow filtration first on a membrane with 0.1-1.2 mm pore size to clarify the product pool and then on a membrane with a separation limit of 5-80 kD to concentrate it, the teachings of which are incorporated herein by reference in its entirety. Similarly, U.S. Patent No. 4,897,465 teaches the concentration and enrichment of a protein such as immunoglobulin from blood serum, egg yolks or whey by sequential ultra filtration on metallic oxide membranes with a pH shift, the teachings of which are incorporated herein by reference in its entirety. Filtration is carried out first at a pH below the isoelectric point (pI) of the selected protein to remove bulk contaminants from the protein retentate, in next adding pH above the pI of the selected protein to retain impurities and pass the selected protein to the permeate. A different filtration concentration method is taught by European Patent No. EP 467 482 B 1 in which de-fatted skim milk is reduced to pH 3-4, below the pI of the milk proteins, to solubilize both casein and whey proteins, the teachings of which are incorporated herein by reference in its entirety. Three successive rounds of ultra filtration are diafiltration and concentrate the proteins to form a retentate containing 15-20% solids of which 90% is protein. Alternatively, British Patent Application No. 2179947 discloses the isolation of lactoferrin from whey by ultra filtration to concentrate the sample, fall by weak cation exchange chromatography at approximately a neutral pH, the teachings of which are incorporated herein by reference in its entirety. No measure of purity is reported in PC Publication No. WO 95/22258, a protein such as lactoferrin is recovered from milk that has been adjusted to high ionic strength by the addition of concentrated salt, followed by cation exchange chromatography, the teachings of which are incorporated herein by reference in its entirety.

In all these methods, milk or a fraction thereof is first treated to remove fats, lipids, and other particular matter that would foul filtration membranes or chromatography medium. The initial fractions thus produce may consist of casein, whey, or total milk protein, from which the protein of interest is then isolated.

PCT Patent Publication No. WO 94/19935 discloses a method of isolating a biologically active protein from whole milk by stabilizing the solubility of total milk proteins with a positively charged agent such as arginine, imidazole or Bis-Tris, the

teachings of which are incorporated herein by reference in its entirety. This treatment forms a clarified solution from which the protein may be isolated for example by filtration through membranes that otherwise would become clogged by precipitated proteins.

- 5 U.S. Serial No. 08/648,235 discloses a method for isolating a soluble milk component such as a peptide in its biologically active form from whole milk or a milk fraction by tangential flow filtration, the teachings of which are incorporated herein by reference in its entirety. Unlike previous isolation methods, this eliminates the need for a first fractionation of whole milk to remove fat micelles, thereby simplifying the process in avoiding losses of recovery of bioactivity. This method may be used in combination with additional purification steps to further remove contaminants and purify the product (e.g., the protein of interest).

EXAMPLE: *CLONING A TRANSGENIC GOAT*

- Donors and recipients used in the following example were dairy goats of the following breeds (mixed or not): Alpine, Saanen, and Toggenburg. All goats were maintained at the Genzyme Transgenics farm in Charlton, Massachusetts. Collections and transfers were completed during the spring and early summer (off-season).

20 *Isolation of Caprine Somatic Cells*

- Caprine fetal fibroblast cell lines used as karyoplast donors were derived from six day 35-40 fetuses produced by artificially inseminating non-transgenic does with fresh collected semen from a transgenic antithrombin III (ATIII) founder buck. An ATIII cell line was chosen since it provides a well characterized genetic marker to the somatic cell lines, and it targets high level expression of a complex glycosylated protein (ATIII) in the milk of lactating does. Three fetuses which were derived from the semen of the transgenic ATIII buck were surgically removed at day 40 post coitus and placed in equilibrated $\text{Ca}^{++}/\text{Mg}^{++}$ -free phosphate buffered saline (PBS). Cell suspensions were prepared by mincing and digesting fetal tissue in 0.025% trypsin/0.5 mM EDTA at 37°C for ten minutes. Cells were washed with equilibrating Medium 199™ (M199)(Gibco) +10% Fetal Bovine Serum (FBS)

supplemented with nucleosides, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 1% penicillin/streptomycin (10,000 I.U. each/ml) (fetal cell medium), and cultured in 25 cm² flasks. The cultures were re-fed 24 hours later with equilibrated fetal cell medium. A confluent monolayer of primary fetal cells was harvested by
5 trypsinization on day four by washing the monolayer twice with Ca⁺⁺/Mg⁺⁺-free PBS, followed by incubation with 0.025% trypsin/0.5 mM EDTA at 38°C for 7 minutes.

Cells potentially expressing ATIII were then prepared for cryopreservation, or maintained as stock cultures.

10 *Sexing and Genotyping of Donor Cell Lines*

Genomic DNA was isolated from fetal head tissue for ATIII donor karyoplasts by digestion with proteinase K followed by precipitation with isopropanol as described in Laird *et al.* (1991) *Nucleic Acid Res.* 19:4293, and analyzed by polymerase chain reaction (PCR) for the presence of human
15 antithrombin III (ATIII) sequences. The ATIII sequence is part of the BC6 construct (Goat Beta-casim - humanATIII cDNA) used to generate the ATIII transgenic line as described in Edmunds *et al.* (1998) *Blood* 91:4561-4571. The human ATIII sequence was detected by amplification of a 367 bp sequence with oligonucleotides GTC11 and GTC12 (see below). For sexing, the zfX/zfY primer pair was used (see
20 below) giving rise to a 445 pb (zfX)/447 bp (sfy) doublet. Upon digestion with the restriction enzyme SacI (New England Biolabs), the zfX band was cut into two small fragments (272 and 173 bp). Males were identified by the presence of the uncut 447 bp zfY band.

For the PCR reactions, approximately 250 ng of genomic DNA was diluted
25 in 50 µl of PCR buffer (20 mM Tris pH 8.3, 50 mM KCl and 1.5 mM MgCl₂, 0.25 mM deoxynucleotide triphosphates, and each primer at a concentration of 600 nM with 2.5 units of Taq polymerase and processed using the following temperature program.

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	1 cycle at	94°C	60 seconds
	5 cycles at	94°C	30 seconds
		58°C	45 seconds
		74°C	45 seconds
5	30 cycles at	94°C	30 seconds
		55°C	30 seconds
		74°C	30 seconds

The following primer set was used to detect the human ATIII sequence:

GTC 11: CTCCATCAGTTGCTGGAGGGTGTCATTA (SEQ ID NO: 1)

10 GTC 12: GAAGGTTTATCTTTTGTCTTGCTGCTCA (SEQ ID NO: 2)

The following primer set was used for sexing:

zfX: ATAATCACATGGAGAGCCACAAGC (SEQ ID NO: 3)

zfY: GCACTTCTTTGGTATCTGAGAAAG (SEQ ID NO: 4)

Two of the fetuses were identified to be male and were both negative for the
 15 ATIII sequence. Another fetus was identified as female and confirmed positive for
 the presence of the ATIII sequence.

Preparation of ATIII-Expressing Donor Cells for Embryo Reconstitution

A transgenic female line (CFF155-92-6) originating from a day 40 fetus was
 identified by PCR analysis, as described above, and used for all nuclear transfer
 20 manipulations. Transgenic fetal fibroblast cells were maintained in 25 cm² flasks
 with fetal cell medium, re-fed on day four following each passage, and harvested by
 trypsinization on day seven. From each passage, a new 25 cm² flasks was seeded to
 maintain the stock culture. Briefly, fetal cells were seeded in 4-well plates with fetal
 cell medium and maintained in culture (5% CO₂ at 39°C). forty-eight hours later,
 25 the medium was replaced with fresh fetal cell medium containing 0.5% FBS. The
 culture was re-fed every 48-72 hours over the next seven days with fresh fetal cell
 medium containing 0.5% FBS. On the seventh day following first addition of fetal

cell medium (0.5% FBS), somatic cells used as karyoplast donors were harvested by trypsinization as previously described. The cells were resuspended in equilibrated M199+10% FBS supplemented with 2mM L-glutamine, 1% penicillin/streptomycin (10,000 I.U. each/ml) one to three hours prior to fusion to the enucleated oocytes.

5 *Karyotyping of Cell Lines*

The clonal lines were further evaluated by karyotyping to determine gross chromosomal abnormalities in the cell lines. Cells were induced to arrest at metaphase by incubation with 0.02 µg/ml of Demecolcine (Sigma) for 12 hours. After trypsinization, the resulting pellet was suspended in a hypotonic solution of 75 mM KCl in water and incubated at 37°C for 20 minutes. Cells were fixed for 5 minutes each time in 3 changes of ice-cold acetic acid-methanol (1:3) solution before drops of the cell suspension were placed on pre-washed microscopic slides. Following air-drying, chromosome preparations were stained with 3% Giemsa stain (Sigma) in PBS for 10 minutes. The chromosome spreads were counted for each cell line at 1000x magnification under oil immersion.

Immunohistochemical Analysis

Antibodies specific for vimentin (Sigma) and pan-cytokeratin (Sigma) were used to characterize and confirm the morphology of the cell lines. Cells were plated in sterile gelatin coated cover slips to 75% confluence and fixed in 2% paraformaldehyde with 0.05% saponin for 1 hour. Cells were incubated in 0.5% PVP in PBS (PBS/PVP) with primary antibodies for 2 hours at 37°C, rinsed with 3 changes of PBS/PVP at 10 minute intervals, and incubated for 1 hour in secondary antibodies conjugated with Cy3 and FITC respectively. Alkaline phosphatase (Sigma) activity of the cells was also performed to determine the presence or absence of undifferentiated cells. The coverslips were rinsed and subsequently mounted on glass slides with 50% glycerol in PBS/PBP with 10 µg/ml bisbenzimidazole (H-33342, Sigma) and observed under fluorescent microscopy.

Epithelial and fibroblast lines positive for vimentin and pan-cytokeratin, respectively, and negative for alkaline phosphatase activity were generated from the ATIII primary cultures. In the cell cultures, two morphologically distinct cell types

were observed. Larger "fibroblast-like" cells stained positive for vimentin and smaller "epithelial-like" cells stained positive for pan-cytokeratin which coexisted in the primary cell cultures. The isolated fibroblast lines from ATIII showed a tendency to differentiate into epithelial-like cells when cultured for 3 days after reaching confluency. Subsequent passages induced selection against fibroblast cells giving rise to pure epithelial cells as confirmed by the lack of positive staining for vimentin. Senescence or possible cell cycle arrest was first observed at passage 28. These cells appear bigger in size ($>30\text{ }\mu\text{m}$) compared to the normally growing cells ($15\text{-}25\text{ }\mu\text{m}$) and can be maintained in culture in the absence of apparent mitotic activity for several months without loss of viability. Embryo reconstruction using nuclei from the arrested cells produced morula stage embryos suggesting reacquisition of mitotic activity.

Donor Karyoplast Cell Cycle Synchronization and Characterization

Selected diploid transgenic female cell lines were propagated, passaged sequentially and cryobanked as future karyoplast stock. Donor karyoplasts for nuclear transfer were seeded in 4 well plates and cultured for up to 48 hours in DMEM + 10% FBS or when cells reached 70-80% confluency. Subsequently, the cells were induced to exit growth phase and enter the quiescent stage (G_0) by serum deprivation for seven days using DMEM supplemented with 0.5% FBS to synchronize the cells. Following synchronization at G_0 , a group of cells were induced to re-enter the cell cycle by resuspending the cells in M199 + 10% FBS up to three hours prior to karyoplast-cytoplast fusion to synchronize the cells at the early G_1 phase prior to START. A second group of cells were also released from the quiescent state and cultured in M199 + 10% FBS for 12 or 36 hours to synchronize cells at the S-phase. Cells were harvested by standard trypsinization and resuspended in M199 + 10% FBS and electrofused as karyoplasts donors within 1 hour.

The metaphase spreads from the transgenic cell lines carrying the ATIII construct at passage 5 was 81% diploid and this did not alter significantly at passage 15 where 78% of the spreads were diploid.

Cell cycle synchrony was determined by immunohistochemical analysis using antibodies against cyclin D1, 2, 3 and PCNA (Oncogene Research Products) for the absence of protein complex to indicate quiescence (G_0) or presence of the complex to indicate G_1 entry. Cells in the presumed S-phase of the cell cycle were identified by the presence of DNA synthetic activity using the thymidine analog 5-bromo 2'-deoxyuridine-5'-triphosphate (BrDu, Sigma) and streptavidin-Biotin BrDu staining kit (Oncogene Research Products).

Immunofluorescence analysis of cells subjected to the synchronization regimen demonstrated that following seven days of serum deprivation, 90% of the cells were negative for G_1 stage cyclins D1, 2, 3 and PNCA, and were therefore in G_0 arrest. Restoration of the serum content to 10% for this line induced reentry into the cell cycle with approximately 74% of the cells reaching early G_1 within 3 hours following serum addition based on positive staining for cyclins D1, 2, 3 and PNCA. Serum restoration for 12 and 36 hours showed that 89% of the cells were positive for BrDu indicating DNA synthetic activity. In this study, clonal lines generally responded differently to the serum synchronization regimen. An indirect relationship was observed where the rate of cell synchronization decreases with the increase in passage numbers. Further, as passage number increased the population doubling times decreased, each clonal cell line revealed a decreased sensitivity to serum synchronization of the cell cycle.

Superovulation of Donor Goats and Oocyte Collection

Estrus was synchronized on day 0 by a 6 mg subcutaneous Norgestomet ear implant (Synchro-mate B). A single injection of prostaglandin (PGF 2α) (Upjohn US) was administered on day 7. Starting on day 12, FSH (Folltropin-V, Vetrepahrm, St. Laurent, Quebec, Canada) was administered twice daily over four consecutive days. The ear implant was removed on day 14. Twenty-four hours following implant removal, the donor animals were mated several times to vasectomized males over a 48 hour interval. A single injection of GnRH (Rhone-Merieux US) was administered intramuscularly following the last FSH injection. Oocytes were recovered surgically from donor animals by mid-ventral laparotomy approximately 18 to 24 hours following the last mating, by flushing the oviduct with

Ca⁺⁺/Mg⁺⁺ -free PBS prewarmed at 37°C. Oocytes were then recovered and cultured in equilibrated M199 + 10% FBS supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin (10,000 I.U. each/ml).

Oocyte Enucleation

5 *In vivo* matured oocytes were collected from donor goats. Oocytes with attached cumulus cells or devoid of polar bodies were discarded. Cumulus-free oocytes were divided into two groups: oocytes with only one polar body evident (metaphase II stage) and the activated telophase II protocol (oocytes with one polar body and evidence of an extruding second polar body). Oocytes in telophase II were
10 cultured in M199 + 10% FBS for 3 to 4 hours. Oocytes that had activated during this period, as evidenced by a first polar body and a partially extruded second polar body, were grouped as culture induced, calcium activated telophase II oocytes (telophase II-Ca²) and enucleated. Oocytes that had not activated were incubated for 5 minutes in PBS containing 7% ethanol prior to enucleation. Metaphase II stage
15 oocytes (one polar body) were enucleated with a 25-30 micron glass pipette by aspirating the first polar body and adjacent cytoplasm surrounding the polar body (approximately 30% of the cytoplasm) presumably containing metaphase plate.

As discussed above, telophase stage oocytes were prepared by two procedures. Oocytes were initially incubated in phosphate buffered saline (PBS, Ca²⁻
20 /Mg²⁺ free) supplemented with 5% FBS for 15 minutes and cultured in M199 + 10% FBS at 38°C for approximately three hours until the telophase spindle configuration or the extrusion of the second polar body was reached. All the oocytes that responded to the sequential culture under differential extracellular calcium concentration treatment were separated and grouped as telophase II-Ca²⁺. The other
25 oocytes that did not respond were further incubated in 7% ethanol in M199 + 10% FBS for 5-7 minutes (telophase II-EtOH) and cultured in M199 + 10% FBS for 2 to 4 hours. Oocytes were then cultured in M199 + 10% FBS containing 5 µg/ml of cytochalasin-B for 10-15 minutes at 38°C. Oocytes were enucleated with a 30
30 micron (OD) glass pipette by aspirating the first polar body and approximately 30% of the adjacent cytoplasm containing the metaphase II or about 10% of the

cytoplasm containing the telophase II spindle. After enucleation the oocytes were immediately reconstructed.

Embryo Reconstruction

CFF 155-92-6 somatic cells used as karyoplast donors were harvested on day 5 7 by trypsinizing (0.025% trypsin/0.5 mM EDTA) (Sigma) for 7 minutes. Single cells were resuspended in equilibrated M199 + 10% FBS supplemented with 2 mM L-glutamine, penicillin/streptomycin. The donor cell injection was carried out in the same medium as for enucleation. Donor cells were graded into small, medium and large before selection for injection to enucleated cytoplasts. Small single cells 10 (10-15 micron) were selected with a 20-30 micron diameter glass pipette. The pipette was introduced through the same slit of the zona made during enucleation and donor cells were injected between the zona pellucida and the ooplasmic membrane. The reconstructed embryos were incubated in M199 30-60 minutes before fusion and activation.

15 *Fusion and Activation*

All reconstructed embryos (ethanol pretreatment or not) were washed in fusion buffer (0.3 M mannitol, 0.05 mM CaCl_2 , 0.1 mM MgSO_4 , 9 mM K_2HPO_4 , 0.1 mM glutathione, 0.1 mg/ml BSA in distilled water) for 3 minutes before electrofusion. Fusion and activation were carried out at room temperature, in a 20 chamber with two stainless steel electrodes 200 microns apart (BTX 200 Embryomanipulation System, BTX-Genetronics, San Diego, CA) filled with fusion buffer. Reconstructed embryos were placed with a pipette in groups of 3-4 and manually aligned so the cytoplasmic membrane of the recipient oocytes and donor CFF155-92-6 cells were parallel to the electrodes. Cell fusion and activation were 25 simultaneously induced 32-42 hours post GnRH injection with an initial alignment/holding pulse of 5-10 V AC for 7 seconds, followed by a fusion pulse of 1.4 to 1.8 KV/cm DC for 70 microseconds using an Electrocult Manipulator and Enhancer 400 (BTX-Genetronics). Embryos were washed in fusion medium for 3 minutes, then they were transferred to M199 containing 5 $\mu\text{g/ml}$ cytochalasin-B 30 (Sigma) and 10% FBS and incubated for 1 hour. Embryos were removed from

M199/cytochalasin-B medium and cocultured in 50 microliter drops of M199 plus 10% FBS with goat oviductal epithelial cells overlaid with paraffin oil. Embryo cultures were maintained in a humidified 39°C incubator with 5% CO₂ for 48 hours before transfer of the embryos to recipient does.

- 5 Reconstructed embryos at 1 hour following simultaneous activation and fusion with G₀, G₁ and S-phase karyoplasts all showed nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) when the cytoplasts were at the arrested metaphase II stage. Subsequent nuclear envelope formation was observed to be at about 35% at 4 hour post activation. Oocytes reconstructed at
- 10 metaphase II stage showed that an average of 22% of oocytes observed at 1 hour post fusion of G₀, G₁ and S-phase karyoplast underwent NEBD and PCC, whereas the remaining oocytes have intact nuclear lamina surrounding the decondensing nucleus. No consistent nuclear morphology other than lack of, or the occurrence of NEBD and PCC was observed between the metaphase and two metaphase
- 15 reconstruction protocols employed. Differences became evident when cloned embryos were observed to have a higher incidence of advanced cleavage stages (8 to 32 blastomeres) when embryos were reconstructed with S-phase donor nuclei compared to when G₀ or G₁ stage karyoplasts were used (2 to 8 blastomeres) following culture *in vitro* for 36 to 48 hours. Fluorescent microscopy analysis
- 20 showed that the nuclei of some of the rapidly dividing embryos were fragmented. Other embryos developed to the 32 to 64 cell stage within 3 days of culture before cleavage development was blocked. Analysis of blastomere and nuclei numbers of these embryos showed the failure of synchronous occurrence of cytokinesis and karyokinesis wherein blastomeres were either devoid or their corresponding nuclei
- 25 or contained multiple nuclei. In contrast, morphologically normal looking embryos showed synchronous cytokinesis and karyokinesis.

Goat Oviductal Epithelial Cells (GOEC) Reconstructed Embryo Co-culture

- GOEC were derived from oviductal tissue collected during surgical oviductal flushing performed on synchronized and superovulated does. Oviductal tissue from
- 30 a single doe was transferred to a sterile 15 ml polypropylene culture tube containing 5 ml of equilibrated M199, 10% FBS, 2 mM L-glutamine, penicillin/streptomycin.

A single cell suspension was prepared by vortex mixing for 1 minute, followed by culture in a humidified 5% CO₂ incubator at 38°C for up to one hour. The tube was vortexed a second time for one minute, then cultured an additional five minutes to allow debris to settle. The top four millimeters containing presumed single cells was
5 transferred to a new 15 ml culture tube and centrifuge at 600x g for 7 minutes, at room temperature. The supernatant was removed, and the cell pellet resuspended in 8 ml of equilibrated GOEC medium. The GOEC were cultured in a 25 cm² flask, re-fed on day 3, and harvested by trypsinization on day six, as previously described. Monolayers were prepared weekly, from primary GOEC cultures, for each
10 experiment. Cells were resuspended in GOEC medium at 5x10⁵/ml, and 50 microliter/well was seeded in 4-231l plates (15mm). The medium was overlaid with 0.5 ml light paraffin oil, and the plates were cultured in a humidified 5% CO₂ incubator at 38°C. The cultures were re-fed on day two with 80% fresh equilibrated culture medium. All reconstructed embryos were cocultured with the GOEC
15 monolayers in vitro in incubator at 39°C, 5% CO₂ before transfer to recipients at GTC farm.

All experimental replicates for ATIII yielded cleavage stage embryos that were transferable on day 2 into synchronized recipients. Embryos using fibroblasts and epithelial cell phenotype as donor karyoplasts showed cleavage and
20 development in culture. The percentage of cleavage development was higher in reconstructed couplets that used preactivated metaphase II stage cytoplasts (45%) and metaphase II-ethanol activated (56%) when compared to cytoplasts used at metaphase II arrested (35%) using ATIII karyoplasts. There were no differences observed in the cleavage rates of embryos that were reconstructed using donor
25 karyoplasts in G₀, G₁ or S-phase of the cell cycle although, the morphological quality of embryos was better when donor karyoplasts were in as G₀ or G₁ compared to S-phase. Embryos were generally between the 2 to 8 cell stage with the majority of the embryos having 3-4 blastomeres at the time of transfer. Normal cleavage development corresponded chronologically to approximately 36 to 48 hours post
30 fusion and activation. Morphologically normal appearing embryos were selected at the 2 to 8 cell stage following development *in vitro* for 36 to 48 hours.

Estrus Synchronization of Recipient Does

Hormonal treatments were delayed by 1 day for recipients (as compared to donor) to insure donor/recipient synchrony. Estrus was synchronized on day 1 by a 6 mg subcutaneous norgestomet ear implant. A single injection of prostaglandin was administered on day 8. Starting on day 14, a single intramuscular treatment of PMSG (CalBiochem US) was administered. The ear implant was removed on day 15. Twenty-four hours following implant removal, recipient does were mated several times to vasectomized males over three consecutive days.

Embryo Transfer to Recipient Does

Reconstructed embryos were co-cultured with GOEC monolayers for approximately 48 hours prior to transfer to synchronized recipients. Immediately prior to transfer, reconstructed embryos were placed in equilibrated Ham's F-12 medium + 10% FBS. Two to four reconstructed embryos were transferred via the fimbria into the oviductal lumen of each recipient. Transfers were performed in a minimal volume of Ham's F-12 medium + 10% FBS using a sterile fire-polished glass micropipet.

The development of embryos reconstructed by nuclear transfer using transgenic caprine fetal fibroblasts and *in vivo* derived oocytes is summarized in Table 1. There was a total of 14 rounds of collection and transfers, with 4 donors set up for collection and 5-6 recipient does set up for transfer 48 hours later. The three different enucleation/activation protocols were employed: Metaphase II, Metaphase, and Metaphase II pretreated with Ethanol. Following fusion-activation, reconstructed embryos were co-cultured with primary goat epithelial cells, at least until cleavage (2-cell stage) up to early 16-cell stage; with most embryos being transferred at chronologically correct 2- and 4-cell stages. All transfers were surgical and oviductal, in hormonally synchronized recipients (due to the season). Rates of development were slightly superior when using Metaphase protocol and Ethanol protocol as compared to the Metaphase II protocol. This is partly due to the fact that enucleation of the second polar body seems less traumatic for the oocytes, and partly due to what seems to be higher activation rate for oocytes pretreated with ethanol.

TABLE 1

Development of caprine embryos reconstructed by nuclear transfer of transgenic fetal fibroblasts. Three enucleation/procedure were used: Metaphase II (first polar body enucleation), Metaphase (second polar body enucleation), Ethanol (preactivation of Metaphase II stage oocytes by 7% ethanol treatment prior to enucleation). In all cases, concomitant fusion and activation was used.

Enucleation and Activation Protocol	Oocytes Reconstructed	Oocytes Lysed (%)	Embryos Cleaved (%)	Embryos Transferred
Metaphase II	138	67 (48.5)	48 (35)	47
Telophase- Ca^{2+}	92	38 (41)	41 (44)	38
Telophase-EtOH	55	23 (42)	31 (56)	27

Following embryo transfer, recipient does were examined by ultrasound, as early as day 25. High pregnancy rates ranging from 55-78% for ATIII recipient does were diagnosed. For all three enucleation/activation protocols, it was observed that high proportion of does (65%) appeared positive at day 30. However, it must be noted that, in most cases, fetal heartbeats could not be detected at such an early stage. Moreover, the positive ultrasound signal detected at day 30 was not characteristic of normal embryo development and appeared closer to vesicular development not associated with the formation of an embryo proper. This kind of embryonic development is not typically observed in other caprine embryo transfer programs (for example with microinjected embryos). Biweekly, examination of these vesicular developments between day 25 and 40 established that these pregnancies were abnormal and at day 40, most of the fetuses were reabsorbed and normal ultrasound images were not apparent.

However, for 2 pregnancies, heartbeats were detected by day 40. In these 2 cases, ultrasound examination between day 25 and day 40, not only detected a heartbeat, but also showed the development of recognizable embryonic structures.

One of these pregnancies was established using the Metaphase II enucleation/activation protocol, fusing the enucleated cytoplasm to a quiescent karyoplast originating from a passage 6 culture of the CFF155-92-6 fibroblast cell line. In this instance, 4 four-cell stage reconstructed embryos were transferred to the oviduct of the recipient doe. The other pregnancy (twins) was obtained from embryos reconstructed according to the Metaphase enucleation/activation protocol, fusing an enucleated cytoplasm derived from preactivated metaphase Ca^{2+} oocytes and G_1 karyoplasts originating from a passage 5 culture of the CFF155-92-6 epithelial cell line. In this case, 3 reconstructed embryos (1 two-cell stage and 2 four-cell stage) were transferred to the oviduct of the recipient doe.

No pregnancies were observed with embryos generated by the Ethanol enucleation/activation protocol. However, numbers are not large enough to conclude on the relative efficacy of the 3 enucleation/activation protocols used in this study.

Table 2.
Induction of pregnancy and further development following transfer of caprine embryos reconstructed with transgenic fetal fibroblasts and activated according to three protocols.

Enucleation Activation Protocol	Recipients (average # of embryos/recipient)	Ultrasound Results (positive/total recip)			Term Pregnancies
		30 days	40 days	50 days	
Metaphase II	15 (3.1)	9/15	1/15	1/15	1
Telophase-Ca ²⁺	14 (2.7)	11/14	1/14	1/14	1 (twins)
Telophase-EtOH	9 (3)	5/9	0/9	0/9	0

Perinatal Care of Recipient Embryos

Does were monitored daily throughout pregnancy for outward signs of health (e.g., appetite, alertness, appearance). Pregnancy was determined by ultrasonograph 25-28 days after the first day of standing estrus. Does were ultrasounded biweekly
5 till approximately day 75 and there after once a month to monitor the assess fetal viability. Additionally, recipient does had serum samples drawn at approximated day 21 post standing estrus for serum progesterone analysis. This was to determine if a functioning corpus luteum was present and how this compared to the animal's reproductive status (i.e., pregnancy). At approximately day 130, the pregnant does
10 were vaccinated with tetanus toxoid and Clostridium C&D. Selenium & vitamin E (Bo-Se) and vitamins A, D, and B complex were given intramuscularly or subcutaneously and a dewormer was administered. The does were moved to a clean kidding stall on approximately day 143 and allowed to acclimate to this new environment prior to kidding. Observations of the pregnant does were increased to
15 monitor for signs of pending parturition. After the beginning of regular contractions, the does remained under periodic observation until birth occurred. If labor was not progressive after approximately 15 minutes of strong contractions the fetal position was assessed by vaginal palpation. If the position appeared normal then the labor was allowed to proceed for an additional 5-30 minutes (depending on
20 the doe) before initiating an assisted vaginal birth. If indicated a cesarean section was performed. When indicated, parturition was induced with approximately 5-10 mg of PGF2 α (e.g. Lutalyse). This induction can occur approximately between 145-155 days of gestation. Parturition generally occurs between 30 and 40 hours after the first injection. The monitoring process is the same as described above.

25 Once a kid was born, the animal was quickly towel dried and checked for gross abnormalities and normal breathing. Kids were immediately removed from the dam. Once the animal was determined to be in good health, the umbilicus was dipped in 7% tincture of iodine. Within the first hour of birth, the kids received their first feeding of heat-treated colostrum. At the time of birth, kids received injections
30 of selenium & vitamin E (Bo-Se) and vitamins A, D, and B complex to boost performance and health.

The first transgenic female goat offspring produced by nuclear transfer was

born after 154 days of gestation following the induction of parturition and cesarean delivery. The birth weight of the offspring was 2.35 kg which is within the medium weight range of the alpine breed. The female twins were born naturally with minimal assistance a month later with a gestation length of 151 days. The birth weights of the twins were both 3.5 kg which are also within the medium weight range for twins of this breed. All three kids appeared normal and healthy and were phenotypically similar for coat color and expressing markings typical of the alpine breed. In addition, all three offspring were similar in appearance to the transgenic founder buck. No distinguishable phenotypic influence from the breed of the donor oocyte (Saanen, Toggenburg breed) or the heterogeneous expression of the fetal genotype was observed.

Transgenic Cloned Goats

In order to confirm that the three kids were transgenic for the BC6 construct comprising the goat beta casein promoter and the human ATIII gene sequence, PCR amplification and southern analysis of the segment of the transgene were performed.

Shortly after birth, blood samples and ear skin biopsies were obtained from the cloned female goats and the surrogate dams. The samples were subjected to genomic DNA isolation. Laird *et al.*, *Nucleic Acids Res.*, 19:4293 (1991). Each sample was first analyzed by PCR using ATIII specific primers, and then subjected to Southern blot analysis using the ATIII cDNA (Edmunds *et al.*, *Blood*, 91:4561-4571 (1998). For each sample, 5 µg of genomic DNA was digested with *EcoRI* (New England Biolabs, Beverly, MA), electrophoresed in 0.7% agarose gels (SeaKam®, ME) and immobilized on nylon membranes (MagnaGraph, MSI, Westboro, MA) by capillary transfer following standard procedures (Laird *et al.*, *Nucleic Acids Res.*, 19:4293 (1991). Membranes were probed with the 1.5 kb *XhoI* to *SalI* ATIII cDNA fragment labeled with α ^{32}P dCTP using the Prime-It® kit (Stratagene, La Jolla, CA). Hybridization was executed at 65°C overnight (Church *et al.*, *Prot. Natl. Acad. Sci. USA*, 81:1991-1995 (1984). The blot was washed with 0.2 X SSC, 0.1% SDS and exposed to X-MAT™ AR film for 48 hours.

PCR analysis confirmed that all of the kids were transgenic for the BC6 construct comprising the goat beta casein promoter and the human ATIII gene

sequence. Southern blot analysis demonstrated the integrity of the BC6 transgene. Hybridization to a diagnostic 4.1 kb *Eco*RI fragment was detected for all three cloned animals, the cell lines and a transgenic positive control, but not for the two recipient does. As expected, due to cross hybridization of the ATIII cDNA probe to
5 the endogenous goat AT locus, a 14 kb band was detected in all samples.

In addition, fluorescence *in situ* hybridization (FISH) was performed to determine the integration site of the BC6 construct. For typing of the cloned goats, whole blood was cultured for lymphocytes harvest (Ponce de Leon *et al.*, *J. Hered.*, 83:36-42 (1992). Fibroblast cells and lymphocytes were pretreated and hybridized
10 as previously described in van de Corput *et al.*, *Histochem Cell Biol.*, 110:431-437 (1998), and Klinger *et al.*, *Am. J. Human. Genet.*, 51:55-65 (1992). A digoxigenin labeled probe containing the entire 14.7 kb BC6 transgene was used in this procedure. The TSA™-Direct system (NEN™ Life Science Products, Boston, MA) was used to amplify the signal. R-bands were visualized using DAPI counterstain
15 and identified as in Di Berardino *et al.*, *J. Hered.*, 78:225-230 (1987). A Zeiss Axioskop microscope mounted with a Hamamatsu Digital Camera was used with Image-Pro® Plus software (Media Cybernetics, Silver Spring, MD) to capture and process images.

FISH analysis of blood cultures from each transgenic kid with probes for the
20 BC6 transgene showed that all three carry a chromosome 5 transgene integration identical to that found in the metaphase plates derived from the CFF6 cell line. Moreover, analysis of the least 75 metaphase plates for each cloned offspring confirmed that they are not mosaic for the chromosome 5 transgenic integration.

As final confirmation that all three kids are derived from the transgenic
25 CFF6 cell line, PCR-RFLP analysis for the very polymorphic MHC class II DRB gene was undertaken. Typing for the second exon of the caprine MHC class II DRB gene was performed using PCR-RFLP Typing as described in Amills *et al.*, *Immunopathol.*, 55:255-260 (1996). Fifteen microliters of nested PCR product was digested with 20 units of *Rsa*I (New England Biolabs, Beverly, MA). Following
30 digestion, restriction fragments were separated at room temperature in a 4 to 20% nondenaturing polyacrylamide gel (MVP™ precast gel, Stratagene, La Jolla, CA) in the presence of ethidium bromide.

As illustrated by the *Rsa*II digests of the DRB gene second exon, the three cloned offspring are identical to each other and identical to the CFF6 donor cell line, whereas the recipient does carry different alleles.

Induction of Lactation and Transgene Expression of Proteins in Milk

5 In order to determine whether the targeted mammary gland specific expression of human ATIII proteins were present in milk, the cloned transgenic prepubertal clones were transiently induced to lactate. At two months of age, the cloned offspring was subjected to a two week hormonal lactation-induction protocol. Hormonal induction of lactation for the CFF6-1 female was performed as described
10 in Ryot *et al.*, *Indian J. Anim. Res.*, 10:49-51 (1989). The CFF6-1 kid was hand-milked once daily to collect milk samples for ATIII expression analyses. All protein analysis methods were described in Edmunds *et al.*, *Blood*, 91:4561-4571 (1998). Concentration of recombinant ATIII in the milk was determined by a rapid reversephase HPLC method using a Hewlett Packard 1050 HPLC (Wilmington, DE)
15 with detection at 214 nm. The ATIII activity was evaluated by measuring thrombin inhibition with a two-stage colorimetric endpoint assay. Western blot analysis was performed with an affinity purified sheep anti-ATIII HRP conjugated polyclonal antibody (Sero Tec, Oxford, UK). Samples were boiled for 30 seconds in reducing sample buffer prior to loading onto a 10-20% gradient gel (Owl Scientific).
20 Electrophoresis was operated at 164 volts (constant) until the dye front ran off the gel.

At the end of the treatment, small milk samples of 0.5 to 10 ml were collected daily for 20 days. The small initial volumes of milk, 0.5 to 1 ml, were typical of the amounts seen in prepubertal female goats hormonally induced to lactate. The
25 volumes increased to 10 ml per day by the time the female was dried off, 25 days after the onset. The concentration and activity of ATIII in several of the samples was evaluated. As previously noted with does from this specific BC6 transgenic cell line, high levels of the recombinant ATIII was detected by Western blot analysis (Edmunds *et al.*, *Blood*, 91:4561-4571 (1998)). The concentration of recombinant
30 ATIII in the milk of the cloned offspring was 5.8 grams per liter (205 U/ml) at day 5, and 3.7 grams per liter (14.6 U/ml) by day 9. These were in line with levels

recorded during the early part of a first natural lactation of does from this BC6 line (3.7 to 4.0 grams per liter).

Healthy transgenic goats were obtained by nuclear transfer of somatic cells to oocytes that were enucleated either in the arrested Metaphase II or the activated
5 Metaphase II-stage. These studies show that serum-starved cells used to generate term pregnancies are likely to undergo a transition following restoration with 10% serum.

Immunofluorescence screening revealed that after 7 days of serum starvation fetal somatic cells were negative for G₁ stage cyclins D1, D2, D3 and PCNA;
10 whereas within 3 hours of 10% FBS serum-activation a majority (e.g. approximately 70%) expressed these markers.

Reconstruction of an enucleated metaphase II arrested oocyte with the transfer of a nucleus from a donor karyoplast synchronized at G₀ or G₁ of the cell cycle following simultaneous fusion and activation mimic the chronological events
15 occurring during fertilization. The successful development to term and birth of a normal and healthy transgenic offspring following the simultaneous fusion and activation protocol is in contrast with procedures employed in other studies that report the requirement for prolonged exposure of donor nuclei to elevated cytoplasmic MPF activity to support chromatin remodeling and reprogramming.
20 See Campbell et al. (1996) *Nature* 380:64-66; Wilmut et al. (1997) *Nature* 385:810-813; Schnieke et al. (1997) *Science* 278:2130-2133; Cibelli et al. (1998) *Science* 280:1256-1258. This result challenges the contention that prolonged remodeling of the somatic nuclei in conditions of elevated MPF activity prior to activation is important for embryonic and fetal development to term. The results also
25 demonstrate that a reconstructed embryo may not have a requirement for prolonged exposure of the donor nucleus to MPF nor are NEBD and PCC entirely requisite events. Rather chromatin remodeling events involving NEBD and PCC are likely permissive effects of MPF activity and, as such, may not be required for the acquisition of developmental competence or totipotency. Instead, these events are
30 likely to serve to facilitate the acquisition of synchronicity between the cytoplasm and the karyoplast. These events may even be detrimental if normal diploidy is not maintained when the donor nuclei are induced to undergo PCC with resultant

chromosome dispersion due to an aberrant spindle apparatus due in part to MPF activity. Therefore, karyoplast and cytoplast synchronization with respect to cell cycle is important, first for maintenance of normal ploidy and, second for the proper induction of genome reactivation and subsequent acquisition of developmental competence of reconstructed embryos.

Further support is provided in the second method where chromatin-intact metaphase II arrested oocytes were activated to reduce MPF activity and induce the oocyte to exit the M-phase and enter the first mitotic cleavage. Approximately 3 hours post-activation, the oocytes were enucleated at telophase stage prior to the onset of G₁ and fused and simultaneously activated with a donor karyoplast in G₁ prior to START of the cycle. In addition, the simultaneous activation and fusion insured that tendencies of non-aged oocytes to revert back to an arrested state were circumvented. Using this paradigm, a normal and healthy set of twin cloned transgenic kids were produced. This procedure inherently provides a homogenous synchronization regimen for the cytoplast to coincide closer with the donor nuclei in G₁ prior to START. Further preactivation of the oocyte induces a decline in cytoplasmic MPF activity, thus inhibiting the occurrence of NEBD and PCC. These results suggest that NEBD and PCC is only facultative for the induction of cytoplast and karyoplast synchrony but necessary for acquisition of proper genome reactivation and subsequent development to term of the nuclear transfer embryo using somatic cell nuclei. These finding further suggest that differentiated cells at the G₀ or G₁ stage function similar to embryonic blastomeres with respect to their ability to acquire totipotency when used in combination with an arrested or an activated recipient cytoplast.

The use of both metaphase II arrested and telophase II cytoplasts provides dual options for cytoplast preparation in addition to providing an opportunity for a longer time frame to prepare the cytoplast. The use of Telophase II cytoplasts may have several practical and biological advantages. The telophase approach facilitates efficient enucleation avoiding the necessity for chromatin staining and ultraviolet localization. Moreover, enucleation at telophase enables removal of minimal cytoplasmic material and selection of a synchronous groups of activated donor cytoplasts. This procedure also allows for the preparation of highly homogenous

group of donor nuclei to be synchronized with the cell cycle of the cytoplasm. When used for embryo reconstruction, these populations showed a higher rate of embryonic development *in vitro*. Thus, reconstructed embryos comprised of a synchronously activated cytoplasm and karyoplast are developmentally competent.

5 In addition to a successful transgenic founder production, nuclear transfer of somatic cells allows for the selection of the appropriate transgenic cell line before the generation of cloned transgenic embryos. This is particularly important in the cases where several proteins are to be co-expressed by the transgenic mammary gland. For example, the availability of several completely identical transgenic
10 females producing recombinant human ATIII will help determine the extent of variation in the carbohydrate structure of this protein, as it is produced by the mammary gland. Thus, it may be feasible to improve the characteristics of the recombinant proteins produced in the transgenic animal system by varying environmental factors (e.g., nutrition) or to increase the milk volume yield of
15 lactation-induction protocols to diminish further the time necessary to obtain adequate amounts of recombinant proteins for pre-clinical or clinical programs.

 The high-level expression of recombinant human ATIII detected in the milk of the CFF6-1 cloned goat illustrates one of the most important aspects of this technology. By combining nuclear transfer with lactation-induction in prepubertal
20 goats, it may be possible to characterize transgenic animals and the proteins they secrete in 8 to 9 months from the time of cell line transfection of milk expression. The amount of milk collected in an induced lactation is not only sufficient to evaluate the recombinant protein yield, but, when mg per ml expression levels are obtained, is adequate for more qualitative analysis (glycosylation, preliminary
25 pharmacokinetics, biological and pharmacological activities). The continued availability of the transfected donor cell line also insures that genetically identical animals can be quickly generated, to rapidly supply therapeutic proteins (with predictable characteristics) for clinical trials.

EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without
5 departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A method of cloning a goat, comprising combining a genome from a caprine donor cell with a caprine oocyte, wherein said oocyte is in a metaphase stage of meiotic cell division, and wherein said oocyte is enucleated, said
5 combination forming a nuclear transfer embryo, said nuclear transfer embryo being suitable for gestation to thereby form said cloned goat.
2. The method of Claim 1, wherein the population of said donor cells to be used for cloning said goat are harvested from a synchronous culture system.
- 10 3. The method of Claim 1, wherein said donor cell is selected from the group consisting of somatic cell, germ cell and stem cell.
4. The method of Claim 2, wherein said synchronous culture system contains at least a majority of donor cells in a specific stage of the mitotic cell cycle.
5. The method of Claim 4, wherein said synchronous culture system contains at
15 least a majority of donor cells in G₁ stage.
6. The method of Claim 4, wherein said donor cell is in G₀ stage.
7. The method of Claim 4, wherein said donor cell is in S phase.
8. The method of Claim 4, wherein said donor cell is in G₂/M phase.
9. The method of Claim 3, wherein said donor cell is an adult somatic cell.
- 20 10. The method of Claim 3, wherein said donor cell is an embryonic somatic cell.

11. The method of Claim 9, wherein said adult donor cell is a fibroblast cell.
12. The method of Claim 9, wherein said adult donor cell is an epithelial cell.
13. The method of Claim 10, wherein said embryonic donor cell is a fibroblast cell.
- 5 14. The method of Claim 10, wherein said embryonic donor cell is an epithelial cell.
15. The method of Claim 1, wherein said oocyte is in metaphase II of meiotic cell division.
16. The method of Claim 1, wherein said oocyte is in an arrested state.
- 10 17. The method of Claim 1, wherein said enucleated oocyte contains an oocyte devoid of a nuclear genome.
18. The method of Claim 1, wherein said enucleated oocyte contains an oocyte with a genome, wherein said genome is incapable of functioning.
- 15 19. The method of Claim 18, further comprising the step of enucleating said oocyte by treating said oocyte with a method selected from the group consisting of X-ray irradiation, laser irradiation and chemical inactivation.
20. The method of Claim 1, wherein said genome of said donor cell is combined with said oocyte by implanting the nucleus of said donor cell into said oocyte.
- 20 21. The method of Claim 20, wherein said genome of said donor cell is combined with said oocyte by microinjecting the nucleus of said donor cell into said oocyte.

22. The method of Claim 1, wherein said genome of said donor cell is combined with said oocyte by fusing the nucleus of said donor cell with said oocyte.
23. The method of Claim 22, wherein fusion between said donor cell and said oocyte is performed by electrofusion.
- 5 24. The method of Claim 1, wherein said fusion between said donor cell and said oocyte is performed by chemical means.
25. The method of Claim 24, wherein said chemical means is selected from the group consisting of PEG, PHA and liposomal fusion.
26. The method of Claim 1, wherein said enucleated oocyte is combined with
10 said donor cell by fusion and is activated.
27. The method of Claim 26, wherein said enucleated oocyte is combined with said donor cell by simultaneous fusion and activation.
28. The method of Claim 26, wherein said fusion is followed by activation.
29. The method of Claim 26, wherein said fusion follows activation.
- 15 30. The method of Claim 26, wherein activation is performed by either chemical or electrical means.
31. The method of Claim 30, wherein said chemical means used for said activation is either an ionophore or ethanol.
32. The method of Claim 1, further including the step of recovering a product
20 from said cloned goat.
33. The method of Claim 32, wherein said product is a protein.

34. The method of Claim 33, wherein said product is contained within said cloned goat's blood.
35. The method of Claim 33, wherein said product is contained within said cloned goat's urine.
- 5 36. The method of Claim 33, wherein said product is contained within said cloned goat's hair.
37. The method of Claim 33, wherein said product is contained within said cloned goat's skin.
38. The method of Claim 33, wherein said product is contained within said cloned goat's mammary gland.
- 10
39. The method of Claim 33, wherein said product is contained within said cloned goat's muscle.
40. The method of Claim 33, wherein said product is contained within said cloned goat's viscera, or a component tissue of said viscera.
- 15 41. The method of Claim 40, wherein said viscera is selected from the group consisting of brain, heart, lung, kidney, pancrease, gall bladder, liver, stomach, eye, colon, small intestine, bladder, uterus and testes.
42. The method of Claim 1, wherein said cloned goat is a male.
43. The method of Claim 1, wherein said cloned goat is a female.
- 20
44. The method of Claim 43, wherein said cloned female goat is induced to lactate.

45. The method of Claim 44, wherein said cloned female goat is naturally induced to lactate.
46. The method of Claim 44, wherein said cloned female non-human is artificially induced to lactate.
- 5 47. The method of Claim 44, wherein milk is obtained from a lactating female goat.
48. The method of Claim 1, wherein said donor cell contains a caprine genome that has been genetically engineered.
49. A method of producing a transgenic goat, comprising combining a genome
10 from a caprine donor cell, wherein said genome has been genetically engineered, with a caprine oocyte, wherein said oocyte is in a metaphase stage of meiotic cell division, and wherein said oocyte is enucleated, said combination forming a transgenic nuclear transfer embryo, said nuclear transfer embryo being suitable for gestation to thereby form said transgenic
15 goat.
50. The method of Claim 49, wherein said oocyte is in metaphase II stage of meiotic cell division.
51. The method of Claim 49, wherein said genome of said donor cell contains a transgenic nucleotide sequence.
- 20 52. The method of Claim 51, wherein said genome of said donor cell is heterozygous for said transgenic nucleotide sequence.
53. The method of Claim 51, wherein said genome of said donor cell is homozygous for said transgenic nucleotide sequence.

54. The method of Claim 51, wherein said transgenic nucleotide sequence is selected from the group consisting of a heterologous nucleotide sequence, and a homologous nucleotide sequence.
55. The method of Claim 54, wherein said transgenic nucleotide sequence is selected from the group consisting of a knockout, knockin or other event which disrupts the expression of a caprine donor cell's gene; and a nucleotide sequence that encodes a protein or fragment thereof.
56. The method of Claim 51, wherein said transgenic nucleotide sequence is selected from the group consisting of a hormone, an immunoglobulin, a plasma protein, an enzyme, a secreted protein, a neuropeptide, a neurotransmitter, a non-secreted protein and a structural protein.
57. The method of Claim 56, wherein said transgenic nucleotide sequence encodes a protein which is selected from the group consisting of α -1 proteinase inhibitor, alkaline phosphatase, angiogenin, extracellular superoxide dismutase, fibrogen, glucocerebrosidase, glutamate decarboxylase, human serum albumin, myelin basic protein, proinsulin, insulin, soluble CD4, lactoferrin, lactoglobulin, lysozyme, lactoalbumin, erythropoietin, tissue plasminogen activator, human growth factor, antithrombin III, prolactin, and α 1-antitrypsin.
58. The method of Claim 51, wherein said transgenic nucleotide sequence is a human nucleotide sequence.
59. The method of Claim 51, wherein said transgenic nucleotide sequence is a non-human animal nucleotide sequence.
60. The method of Claim 59, wherein said human nucleotide sequence contains an operatively linked promoter nucleotide sequence.

61. A method of producing a heterologous protein in a goat comprising the steps of:
 - (a) forming a nuclear transfer embryo by introducing a genetically engineered caprine genome which contains a transgene encoding a heterologous protein, into a caprine oocyte, wherein said oocyte is enucleated, and wherein said oocyte is in a metaphase stage of meiotic cell division, thereby forming said nuclear transfer embryo capable of producing said heterologous protein, whereby said nuclear transfer embryo can be implanted into a suitable female goat where it undergoes gestation to form said goat that produces said heterologous protein, and
 - (b) recovering said heterologous protein from said goat.
62. The method of Claim 61, wherein said oocyte is in metaphase II stage of meiotic cell division.
63. The method of Claim 61, wherein said transgene is operatively linked to a promoter nucleotide sequence.
64. The method of Claim 63, wherein said promoter is a host endogenous promoter.
65. The method of Claim 63, wherein said promoter is an exogenous promoter.
66. The method of Claim 63, wherein said promoter is a tissue-specific promoter.
67. The method of Claim 66, wherein said tissue-specific promoter is selected from the group consisting of mammary-specific promoter, blood-specific promoter, muscle-specific promoter, neural-specific promoter, skin-specific promoter, hair-specific promoter and urinary-specific promoter.

METHODS OF CLONING GOATS USING METAPHASE II OOCYTES

ABSTRACT OF THE DISCLOSURE

The present invention is directed to methods for cloning goats. The instant invention is based in part on the discovery that cloned and transgenic goats can be produced by the introduction of a caprine donor cell's nuclear genome into an enucleated caprine oocyte that is in a metaphase stage of meiotic cell division. In one embodiment of the invention, a method of cloning a goat by combining a genome from a caprine donor cell with a enucleated caprine oocyte that is in a metaphase stage of meiotic cell cycling in the production of a nuclear transfer embryo which is suitable for gestation is described.